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Induction of Tyrosine Phosphorylation and Na^+/H^+ Exchanger Activation during Shrinkage of Human Neutrophils*

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The ubiquitous isoform of the Na^+/H^+ exchanger (NHE1) is essential for the regulation of cellular volume. The underlying molecular mechanism, which is poorly understood, was studied in human polymorphonuclear leukocytes (PMN). Suspension of PMN in hypertonic media induced rapid cellular shrinkage and activation of NHE1, which is measurable as a cytosolic alkalinization. Concomitantly, hypertonic stress also induced extensive tyrosine phosphorylation of several proteins. Pretreatment of PMN with genistein, a tyrosine kinase inhibitor, prevented not only the tyrosine phosphorylation in response to a hypertonic shock but also the activation of NHE1. The signal elicited by hyperosmolarity that induces activation of tyrosine kinases and NHE1 was investigated. Methods were devised to change medium osmolarity without altering cell volume and vice versa. Increasing medium and intracellular osmolarity in normovolemic cells failed to activate tyrosine kinases or NHE1. However, shrinkage of cells under iso-osmotic conditions stimulated both tyrosine phosphorylation and NHE1 activity. These findings imply that cells detect alterations in cell size but not changes in osmolarity or ionic strength. The identity of the proteins that were tyrosine-phosphorylated in response to cell shrinkage was also investigated. Unexpectedly, the mitogen-activated protein kinases SAPK, p38, *erk1*, and *erk2* were not detectably phosphorylated or activated. In contrast, the tyrosine kinases $p59^{fgr}$ and $p56/59^{hck}$ were phosphorylated and activated upon hypertonic challenge. We propose that cells respond to alterations in cell size, but not to changes in osmolarity, with increased tyrosine phosphorylation, which in turn leads to the activation of NHE1. The resulting changes in ion content and cytosolic pH contribute to the restoration of cell volume in shrunken cells.

The Na^+/H^+ exchanger isoform 1 (NHE1)[1] is a ubiquitously expressed cation antiporter that is involved in the regulation of cell volume and intracellular pH (pH_i). NHE1 is nearly quiescent in resting cells but becomes activated upon cytosolic acidification or by treatment of the cells with a variety of hormones and growth factors (see Ref. 1 for review). Phosphorylation of the exchanger was suggested to induce its activation, since treatment with growth promoters was found to increase the phosphoserine content of NHE1 (2, 3). Moreover, increased phosphorylation and functional activation were also induced by inhibitors of Ser/Thr phosphatases, such as okadaic acid (3).

NHE1 is also rapidly stimulated when cells are made to shrink in hypertonic solutions (4). It is unclear whether increased osmolarity or reduced cell volume are the signals that trigger activation of the exchanger. The osmotic stimulation of Na^+/H^+ exchange requires intracellular ATP and is not additive with that induced by growth factors (5). These observations suggested that phosphorylation was also involved in the osmotic activation of NHE1. However, the phosphorylation state of the exchanger was found to be unaffected during osmotic challenge (4). Moreover, osmotic stimulation could still be observed following truncation of all the putative phosphorylation sites of NHE1 (6). Thus, the mechanism responsible for osmotically induced stimulation of the exchanger remains unclear. It is possible that phosphorylation of ancillary regulatory proteins is involved. In this context, calcineurin B homolog protein (CHP), a substrate of Ser/Thr kinases, was reported to bind to the cytosolic tail of the antiporter (7). Also, a polypeptide of ~24 kDa, the approximate size of CHP, is constitutively associated with NHE1 in several cell types (8).

Osmotic shrinkage of mammalian cells is a powerful stimulant of MAPK including the stress kinases p38 and SAPK (JNK) (9, 10) and in some instances Erk (11). MAPK have recently been invoked as possible regulators of the activity of NHE1 in platelets (12) and fibroblasts (13) treated with various agonists. The precise mechanism whereby shrinkage stimulates the kinases is unknown, as is their relationship to the osmotic stimulation of NHE1.

In this report, we investigated the relationship between the stimulation of protein kinases and the activation of NHE1, and we attempted to determine whether reduced cell volume or increased cytosolic osmolarity were the signals leading to the activation of these effectors. To this end we used human blood neutrophils, which express NHE1 (14) and are known to respond vigorously to changes in medium osmolarity (15).

EXPERIMENTAL PROCEDURES

Materials—Dextran T-500 and Ficoll-Paque were from Pharmacia Biotech Inc. Genistein and erbstatin analog were from Calbiochem. BCECF was from Molecular Probes Inc. Nystatin was from Sigma and was freshly dissolved in dimethyl sulfoxide before each experiment. All other chemicals used were of the highest purity available. The enhanced chemiluminescence detection system and horseradish peroxidase-coupled anti-rabbit and anti-mouse antibodies were from Amer-

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[1] The abbreviations used are: NHE1, Na^+/H^+ exchanger isoform 1; BCECF, 2′,7′-*bis*-(carboxyethyl)5-(6)-carboxyfluorescein; Erk, extracellular signal-regulated kinase; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; LSB, Laemmli sample buffer; MAPK, mitogen-activated protein kinase; PMN, polymorphonuclear leukocytes; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; PVDF, polyvinylidene difluoride; SAPK, stress-activated protein kinase.

sham Corp. Phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology Inc. Polyclonal anti-paxillin antibody was from Zymed Inc. and anti-c-*cbl* was from Transduction Laboratories Inc. Polyclonal antibody against p38 was the generous gift of Dr. Brent Zanke (Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Canada). MAPKAPK-2 polyclonal antibody was the kind gift of Dr. Steven L. Pelech (Kinetek Pharmaceuticals Inc., Vancouver, British Columbia, Canada). A GST-c-*Jun* construct was provided by Dr. James Woodgett (Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Canada). Phospho-specific *Erk* polyclonal antibody was from New England Biolab. *lyn*, *fgr*, and *hck* polyclonal antibodies were generously provided by Dr. Joseph B. Bolen (DNAX Research Institute, Palo Alto, CA).

Solutions—Bicarbonate-free RPMI 1640 was buffered to pH 7.4 with 10 mM Hepes. Isotonic NaCl buffer contained (in mM) 5 KCl, 10 glucose, 140 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, pH 7.4. Isotonic KCl buffer contained 10 glucose, 145 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 Hepes, pH 7.4. Hypertonic NaCl buffer contained 5 KCl, 10 glucose, 240 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 Hepes, pH 7.4. Hypertonic KCl buffer was similar to hypertonic NaCl buffer, except that NaCl was replaced with KCl. Hypotonic NaCl buffer contained 5 KCl, 10 glucose, 50 NaCl 1 $CaCl_2$, 1 $MgCl_2$, and 10 Hepes, pH 7.4. Iso-osmotic sucrose buffer contained 5 KCl, 10 glucose, 280 sucrose, 1 $CaCl_2$, 1 $MgCl_2$, and 10 Hepes, pH 7.4. Ca^{2+} and Mg^{2+} were omitted from all buffers that were used during permeabilization with nystatin. The iso-osmolar buffers were adjusted to 290 ± 5 mOsm with either water or the major salt. All buffers used for cell incubations were nominally HCO_3^--free. Laemmli sample buffer (LSB) contained 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 0.025% bromphenol blue, 62.5 mM Tris, pH 6.8. Nonidet P-40 buffer contained 1% Nonidet P-40, 1 mM EGTA, 150 mM NaCl, and 50 mM Tris, pH 8.0.

Cells—Human PMN were isolated from fresh blood drawn by venipuncture into heparinized tubes. Isolation of cells was performed using dextran sedimentation and centrifugation on Ficoll-Paque cushions as described previously (16). Cells were resuspended in Hepes-buffered RPMI 1640 and kept on a rotary shaker at room temperature until use. When immunoprecipitation was performed, PMN were pretreated with 1 mM diisopropylfluorophosphate for 30 min to minimize proteolysis. Cell volume and counts were assessed with a Coulter Counter (model ZM) equipped with a Channelyzer.

Immunoprecipitation and Immunoblotting—Treatments were stopped by the addition of 2 volumes of ice-cold buffer of the corresponding osmolarity, and the PMN were rapidly sedimented in a microcentrifuge. For experiments where whole cell anti-phosphotyrosine blotting was performed, the cell pellet was resuspended in hot LSB and boiled for 10 min. For immunoprecipitation, the cell pellet was dissolved in ice-cold Nonidet P-40 buffer containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μM sodium vanadate, and 1 mM NaF) and kept on ice for at least 10 min. Immunoprecipitation and immunoblotting were performed as described previously (17). Samples were subjected to 10% SDS-PAGE, transferred to poly(vinylidene difluoride) membranes, and blotted with the appropriate antibody.

Kinase Assays—Tyrosine kinase activity was assayed *in vitro* using enolase as the substrate, as described previously (18). SAPK assays using GST-c-*jun* as a substrate were performed essentially as described (19). Reaction products were separated by 10% SDS-PAGE, and incorporated ^{32}P was quantified with a PhosphorImager equipped with ImageQuant software (Molecular Dynamics Inc.).

Intracellular pH Measurements—PMN (10^7/ml) were incubated with 2 μM of the acetoxymethyl form of BCECF for 15 min at 37 °C, sedimented, and resuspended (at 2×10^7/ml) in the appropriate buffer. Where indicated, the cells were pretreated with nystatin (50 μg/ml) to increase the permeability of the plasmalemma to small monovalent ions (see "Results"). An aliquot of the cell suspension (10^6 cells) was added to 1 ml of prewarmed (37 °C) buffer of the required osmolarity in the cuvette compartment of a spectrofluorimeter (Perkin-Elmer model 650-40). Measurements of BCECF emission and the calibration of fluorescence *versus* pH_i were performed as described previously (20).

Statistical Analysis—All experiments were performed at least in triplicate. Data are presented as means ± S.E. or illustrated as representative traces or blots. Significance was assessed using Student's paired *t* test. A score of $p < 0.05$ was considered significant.

RESULTS

Correlation between Tyrosine Phosphorylation and NHE1 Activation

We tested the effect of hypertonic solutions on PMN volume, measured electronically, and pH_i, estimated from the fluorescence of BCECF. Increasing the osmolarity of the medium from 290 to 475 mOsm by addition of 100 mM NaCl caused a rapid reduction of median cell volume from 327 ± 3 fl to 273 ± 2 fl (means ± S.E., $n = 5$, $p < 0.01$).[2] As shown in Fig. 1*A*, hypertonic stress also induced an alkalinization of the cytosol ranging from 0.2 to 0.3 pH units, which was evident at 30 s and stabilized within 5 min. As in other cells, this alkalinization was mediated by the NHE, since it was abolished by omission of external Na^+ (not shown) or by addition of the specific inhibitor compound HOE694 (see below).

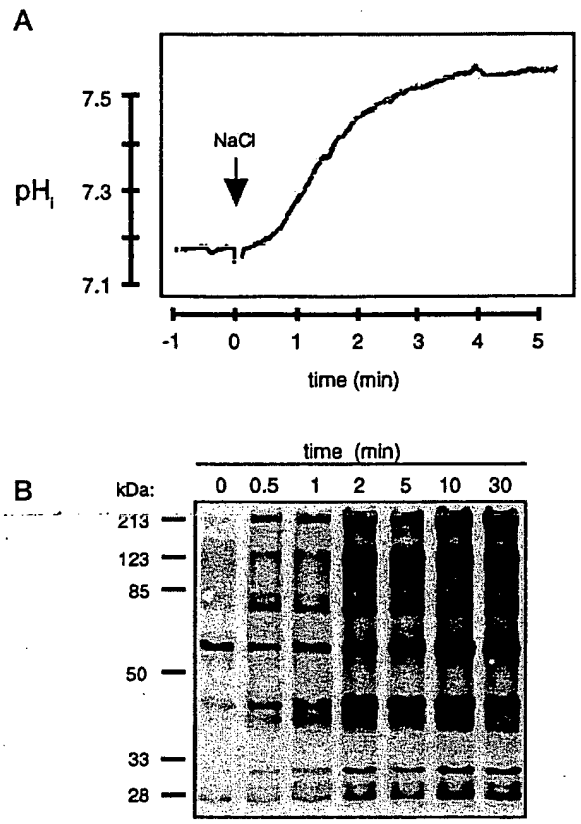


FIG. 1. **Correlation between cytosolic alkalinization and tyrosine phosphorylation in response to hypertonicity.** *A*, BCECF-loaded PMN were suspended in isotonic NaCl medium, and pH_i was monitored fluorimetrically, as described under "Experimental Procedures." Where indicated by the *arrow* the medium was made hypertonic by addition of an extra 100 mM NaCl. *B*, PMN were suspended in isotonic NaCl medium, and at 0 min the medium was made hypertonic by addition of an extra 100 mM NaCl. Samples were then collected after increasing lengths of time, subjected to SDS-PAGE, and transferred to PVDF membranes. Tyrosine-phosphorylated proteins were detected by blotting with anti-phosphotyrosine antibody (4G10). Results in *A* and *B* are representative of three separate experiments.

Phosphorylation of tyrosine residues is one of the earliest events in a variety of signaling cascades. We questioned whether tyrosine phosphorylation was also involved in signaling the osmotic activation of NHE1. To address this possibility, the content of tyrosine-phosphorylated proteins was analyzed by immunoblotting in PMN subjected to hypertonic stress. Fig. 1*B* shows that osmotic shrinkage was associated with a remarkable increase in the phosphotyrosine content of several

[2] Note that PMN shrink in response to osmolarity less than predicted for a perfect osmometer by the Van't Hoff relationship. This is due to the presence of a sizable osmotically unresponsive volume within the cells, likely the secretory granules and/or the nucleus.

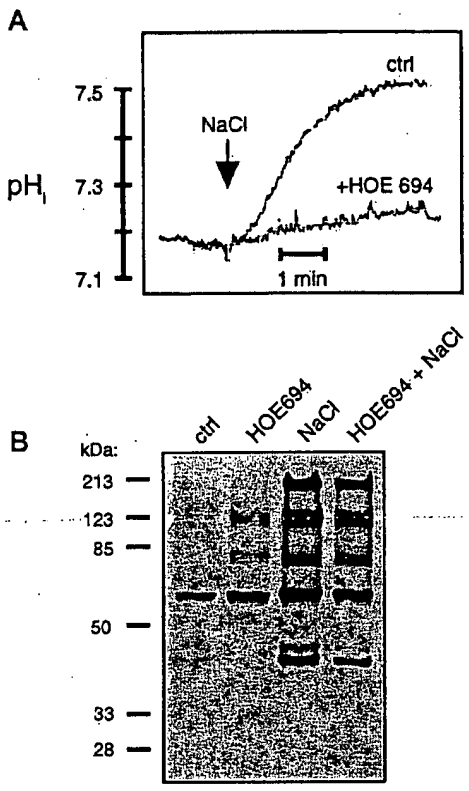


FIG. 2. **Effect of NHE1 inhibition on tyrosine phosphorylation in response to a hypertonic shock.** *A*, BCECF-loaded PMN were suspended in isotonic NaCl medium in the presence or absence of 2 μM HOE694, and pH_i was monitored fluorimetrically, as described under "Experimental Procedures." Where indicated by the *arrow* the medium was made hypertonic by addition of an extra 100 mM NaCl. *B*, PMN in isotonic NaCl medium was pretreated with or without 2 μM HOE694 for 5 min and then stimulated with hypertonic NaCl (100 mM) for 5 min. Samples were collected before (*ctrl*, HOE694) or after addition of NaCl (*NaCl*, NaCl+HOE694), subjected to SDS-PAGE, and transferred to PVDF membranes. Tyrosine-phosphorylated proteins were detected by immunoblotting as in Fig. 1. Results in *A* and *B* are representative of three separate experiments.

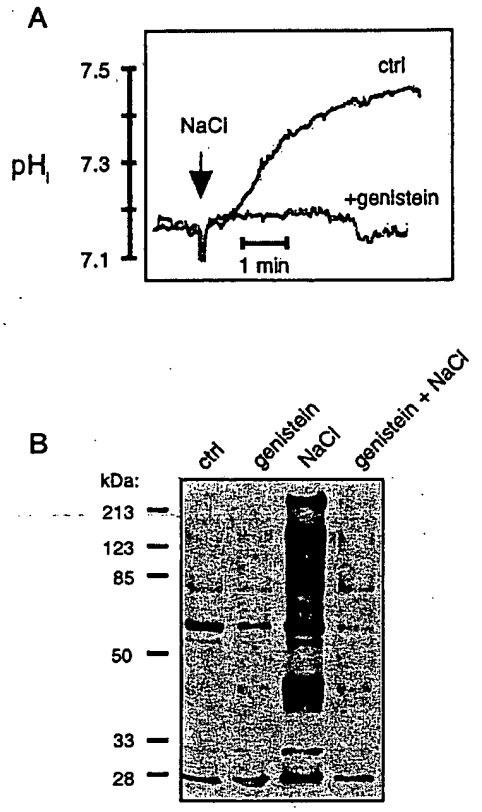


FIG. 3. **Effect of tyrosine kinase inhibition on the activation of NHE1 and on phosphorylation induced by hypertonic shock.** *A*, BCECF-loaded PMN in isotonic NaCl medium were pretreated with or without 100 μM genistein for 30 min, and pH_i was then monitored as in Fig. 1. Where indicated by the *arrow*, the cells were subjected to a hypertonic shock (100 mM NaCl). *B*, PMN in isotonic NaCl medium were pretreated with or without 100 μM genistein for 30 min and then stimulated with hypertonic NaCl (100 mM) for 5 min at 37 °C. Samples were collected before (*ctrl*, genistein) or after addition of NaCl (*NaCl*, NaCl + genistein), subjected to SDS-PAGE, and transferred to PVDF membranes. Tyrosine-phosphorylated proteins were detected by immunoblotting as in Fig. 1. Results in *A* and *B* are representative of three separate experiments.

proteins, which was clearly apparent at 30 s, attained maximum levels by 2 min, and persisted for up to 30 min. Polypeptides of ~210, 125, 74, 60, 42, and 40 kDa were consistently tyrosine-phosphorylated in all of our experiments.

We next investigated whether tyrosine phosphorylation was a consequence or the cause of NHE1 activation. To determine if activation of Na^+/H^+ exchange was required for induction of tyrosine phosphorylation, PMN were pretreated with 2 μM HOE694, a concentration predicted to produce almost complete inhibition of NHE1 (21) and subjected to a hypertonic shock. As shown in Fig. 2, while the inhibitor largely eliminated the cytosolic alkalinization, the accumulation of phosphotyrosine induced by hyperosmolarity was unaffected. A comparable degree of tyrosine phosphorylation was also obtained in cells suspended in a hypertonic KCl (Na^+-free) medium. The absence of Na^+, the external substrate for NHE, precluded cytosolic alkalinization (results not shown). These experiments imply that stimulation of tyrosine phosphorylation by hyperosmolar solutions is not a consequence of activation of NHE1.

We therefore considered whether tyrosine phosphorylation was instead the cause of NHE1 activation. Cells were pretreated with 100 μM genistein, a potent tyrosine kinase inhibitor, and subjected to hypertonicity. Under these conditions, both the cytosolic alkalinization (Fig. 3*A*) and tyrosine phosphorylation were inhibited (Fig. 3*B*). Similar results were obtained by pretreating PMN with 10 μg/ml erbstatin analog, a structurally unrelated tyrosine kinase inhibitor (not shown). These findings suggest that phosphotyrosine accumulation is required for the hypertonic activation of NHE1.

Role of Osmolarity in the Induction of Tyrosine Phosphorylation

We next investigated the signal that triggers phosphotyrosine accumulation in cells exposed to hypertonic media. In principle, the response could be initiated by osmosensors that detect the change in medium or intracellular tonicity. Alternatively, the signal for phosphorylation could be the cellular shrinkage that results from the net loss of cytosolic water. The experiments described below were designed to discern between these alternative models.

Fig. 4*A* illustrates the protocol used to increase the intracellular osmolarity while keeping the cellular volume constant. PMN were suspended in isotonic KCl medium and treated with 50 μg/ml nystatin, a pore-forming molecule that allows the

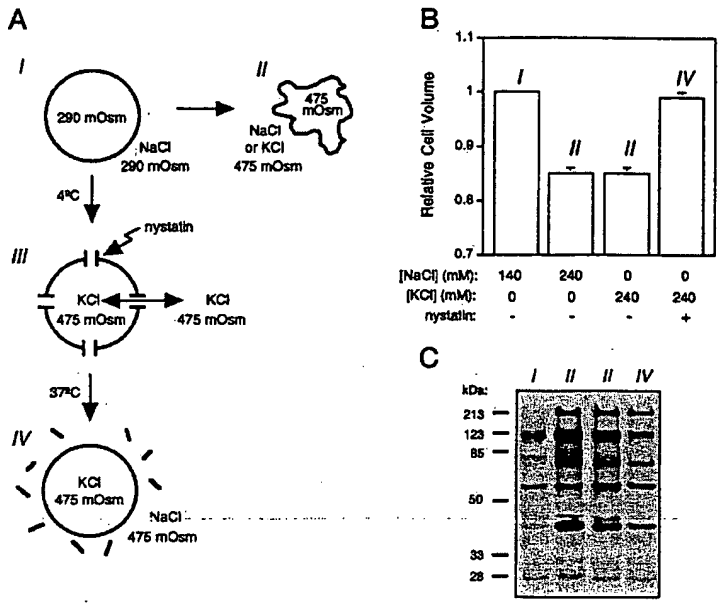


FIG. 4. **Effect of increased osmolarity at constant cell volume on tyrosine phosphorylation.** *A*, diagrammatic representation of the experimental protocols used to either shrink cells (*II*) or to increase intra- and extracellular osmolarity while maintaining cell volume constant, using nystatin (*III* and *IV*). *I*, cells were initially in isotonic NaCl buffer; *II*, cells transferred to hypertonic KCl or NaCl buffer (475 mOsm) which causes shrinkage; *III*, cells resuspended an ice-cold isotonic KCl buffer (115 mM KCl + 50 mM sucrose) containing 50 μg/ml nystatin. After 9 min, an extra 125 mM KCl was added, making the medium hyperosmotic (475 mOsm); *IV*, the cells were then suspended in prewarmed (37 °C) hyperosmolar NaCl buffer (475 mOsm). *B*, PMN were treated under the conditions indicated at the foot of the figure, using the protocols detailed in *A*. The median volume of the cells was then measured after 5 min using the Coulter Counter. The *roman numerals* in *B* and *C* identify the conditions with the diagram in *A*. Data are means ± S.E. of three experiments, counting a minimum of 2×10^4 cells per experiment. Data are normalized to the volume of untreated PMN in isotonic solution (290 mOsm), which averaged 327 ± 3 fl. *C*, cells were treated as in *B* except that aliquots of the suspension were boiled in LSB buffer and used for immunoblotting with anti-phosphotyrosine antibody. Results are representative of three separate experiments.

passage of small monovalent ions across the plasma membrane (22). Sucrose (50 mM), which cannot permeate through nystatin, was added to the medium to prevent swelling due to the presence of impermeant osmolytes within the cells (20). After 9 min, the time required for adequate permeabilization, an additional 125 mM KCl was introduced to render both the extracellular and intracellular solutions hyperosmotic. Because both K^+ and Cl^- permeate readily through nystatin, cell shrinkage is minimal (*step III* in Fig. 4*A*). Cells were then washed at 37 °C to remove extracellular as well as membrane-associated nystatin, resulting in rapid and effective resealing of the membrane, and the hypertonic KCl was replaced with hypertonic NaCl (*step IV*). Sizing with the Coulter-Channelyzer confirmed that, following nystatin treatment in the hypertonic buffer, the volume of the cells was similar to that of untreated PMN in isotonic solution (*cf. columns I* and *IV* in Fig. 4*B*). This contrasts with the shrinkage noted when cells were suspended in hypertonic NaCl or KCl in the absence of nystatin[2] (*II* in Fig. 4, *A* and *B*). Fig. 4*C* confirms that cell shrinkage induced by the hypertonic media (in the absence of nystatin) stimulated tyrosine phosphorylation of multiple proteins, regardless of the solute used (*lanes labeled II* in Fig. 4*C*). By contrast, exposure to hyperosmotic solutions under conditions where shrinkage was prevented (*i.e.* in nystatin-treated cells) resulted in a substantially lower level of tyrosine phosphorylation (*cf. lane IV*). It is noteworthy that the residual increase in phosphorylation may have been caused by a transient shrinkage of the cells that likely occurred when the osmolarity of the medium was raised. Despite the presence of nystatin, some efflux of water from the cells may have preceded entry and equilibration of hyperosmotic KCl into the cells.

We also used a second approach to assess the effect of increasing the osmolarity on protein tyrosine phosphorylation in the absence of significant cell volume changes. For these experiments osmolarity was increased adding 200 mM urea, a rapidly permeating solute, to cells suspended in isotonic NaCl buffer. Fig. 5*A* illustrates the protocol used. The addition of urea did not alter the steady state volume of the cells (measured after 5 min; *II* in Fig. 5), which contrasts with the sustained shrinkage induced by an equimolar concentration of sucrose (*IV* in Fig. 5, *A* and *B*) or 100 mM NaCl (*e.g.* Fig. 4). In parallel experiments, tyrosine phosphorylation was assessed in cells exposed to hyperosmotic urea and was found to be similar to that of cells maintained in isotonic NaCl medium throughout (Fig. 5*C*). That urea did not exert an inhibitory effect on tyrosine phosphorylation was tested by treating cells with either 200 mM sucrose or 100 mM NaCl in the presence (*III* in Fig. 5*A*) or absence (*IV* in Fig. 5*A*) of urea. As shown in Fig. 5*B*, the impermeant osmolytes induced shrinkage both in the presence and absence of urea. More importantly, both sucrose (*cf. lanes 3* and *4* in Fig. 5*C*) and NaCl (*cf. lanes 5* and *6*) activated tyrosine phosphorylation to comparable degrees whether urea was present or not. These results suggest that urea does not *per se* prevent phosphotyrosine accumulation and that an increase in the osmolarity of the medium and/or the intracellular space is not sufficient to induce tyrosine phosphorylation of PMN proteins.

Role of Cell Shrinkage in the Induction of Tyrosine Phosphorylation

In the next series of experiments, we analyzed the contribution of cell volume changes to the induction of tyrosine phosphorylation. To this end, we attempted to induce cell shrinkage while maintaining iso-osmolar conditions. Fig. 6*A* illustrates

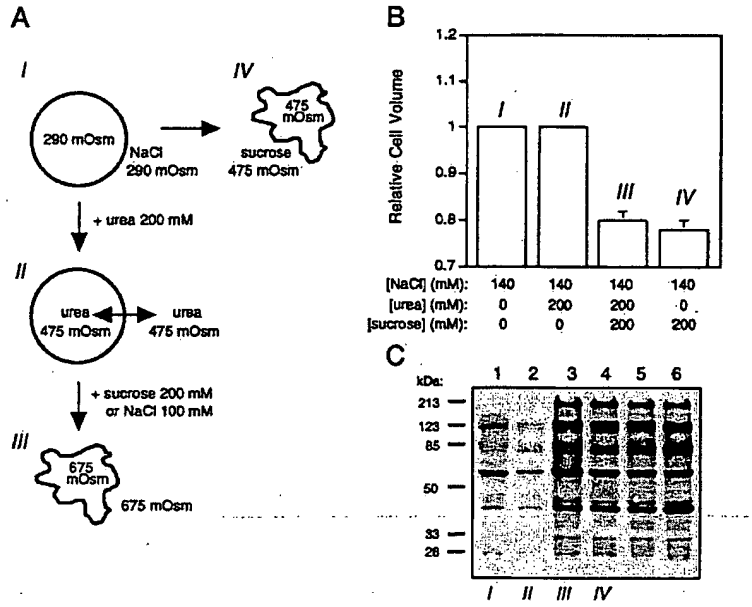


FIG. 5. **Effect of hyperosmolar urea on tyrosine phosphorylation.** *A*, diagrammatic representation of the experimental protocols used to either shrink cells (*IV*), to increase intra- and extracellular osmolarity while maintaining cell volume constant, or using urea (*II*) to shrink cells in the presence of urea (*III*). *I*, cells were initially in isotonic NaCl buffer (290 mOsm); *II*, 200 mM urea was added to increase osmolarity (475 mOsm). Urea rapidly equilibrated across the membrane, with no shrinkage in the steady state; *III*, 200 mM sucrose or 100 mM NaCl was then added to the urea-containing suspension, inducing sustained shrinkage; *IV*, cells transferred directly to medium made hypertonic (475 mOsm) with sucrose. *B*, PMN were treated under the conditions indicated at the foot of the figure, using the protocols detailed in *A*. The median volume of the cells was then measured using the Coulter Counter. The *roman numerals* in *B* and *C* identify the conditions with the diagram in *A*. Data are means ± S.E. of three experiments, normalized to the volume of untreated PMN in isotonic solution; *C*, tyrosine phosphorylation was assessed under the conditions described in *A*. *Lanes 1–4* correspond to conditions *I–IV* in *A* and *B*. *Lanes 5* and *6* are identical to *lanes 3* and *4*, respectively, except that sucrose was replaced with 100 mM NaCl. Results are representative of three separate experiments.

the first method used; PMN were resuspended in an ice-cold iso-osmotic sucrose medium and permeabilized with nystatin for 10 min. While the extracellular sucrose is unable to diffuse through the nystatin pores, intracellular KCl readily diffuses out of the cells (*II* in Fig. 6*A*). The net efflux of KCl is accompanied by osmotically obliged water, thus causing a reduction in cell volume (*cf. I* and *III* in Fig. 6, *A* and *B*). Interestingly, the resulting shrinkage of nystatin-permeabilized PMN in the isotonic sucrose buffer caused a marked increase in tyrosine phosphorylation (Fig. 6*C*, *lane 4*), which was in fact greater than that caused by hypertonic NaCl buffer (*cf. lane 5*). The effect of sucrose was not due to the reduction in ionic strength, since in the absence of nystatin tyrosine phosphorylation was not stimulated. As expected, cell volume was unaffected under these conditions (Fig. 6*B*). Moreover, the stimulation of phosphorylation was not due to nystatin itself, because cells treated with the pore former under conditions intended to keep cell volume constant (125 mM NaCl plus 50 mM sucrose; see Fig. 6*B*) did not show increased phosphorylation. It is also noteworthy that treatment with nystatin in isotonic NaCl buffer, which induced cell swelling (Fig. 6*B*), decreased tyrosine phosphorylation below the level noted in untreated (isotonic) cells (Fig. 6*C*, *cf. lanes 1* and *2*).

A second method used to dissociate the effects of cell shrinkage and hypertonicity is illustrated in Fig. 7*A*. PMN were suspended in hypotonic NaCl buffer (≈50% of the normal osmolarity), thereby causing the cells to swell (*II* in Fig. 7, *A* and *B*). This initial passive swelling was followed by a gradual loss of volume, reaching near normal size after approximately 30 min (*III* in Fig. 7, *A* and *B*). This secondary volume loss, known as regulatory volume decrease, is thought to be mediated by increased permeability to K^+ and anions (23). Subsequent addition of 90 mM NaCl to the medium, which restored the osmolarity to the initial (iso-osmotic, 290 mOsm) level, caused the cells to shrink (*IV* in Fig. 7). Such shrinkage under iso-osmotic conditions was accompanied by a marked phosphotyrosine accumulation, usually exceeding that induced by comparable hypertonic shrinkage (Fig. 7*C*). The combined results of Figs. 6 and 7 demonstrate that tyrosine phosphorylation can be promoted in PMN by reducing the volume of the cells, regardless of the osmolarity of the medium or cytosol.

Role of Cell Volume and Hypertonicity in the Activation of NHE1

The preceding data indicate that tyrosine phosphorylation was triggered by a reduction of the cell volume and not by hypertonicity *per se*. It was therefore of interest to define whether cell volume, as opposed to medium osmolarity, is responsible for activation of NHE1. Protocols like those employed above were used to differentially alter cell volume and osmolarity while measuring pH_i to evaluate the state of activation of NHE1. Fig. 8 shows that a significant cytosolic alkalinization, comparable to that observed during hypertonic stress, was caused by reducing cell volume isotonically using nystatin/sucrose, or by restoring iso-osmolarity after regulatory volume decrease. Conversely, increasing osmolarity while keeping the volume constant, using either nystatin/KCl or urea, failed to activate the antiporter. This pattern correlates closely with that of tyrosine phosphorylation and is consistent with the notion that NHE1 activation lies downstream of phosphotyrosine accumulation.

Identity of Tyrosine-phosphorylated Proteins in Shrunken PMN

MAPK—Because the activation of NHE1 appears to be dependent on phosphotyrosine accumulation, we tried to identify

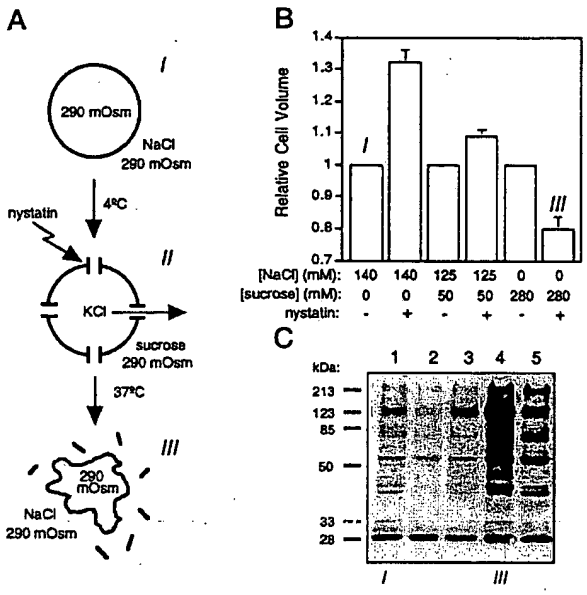
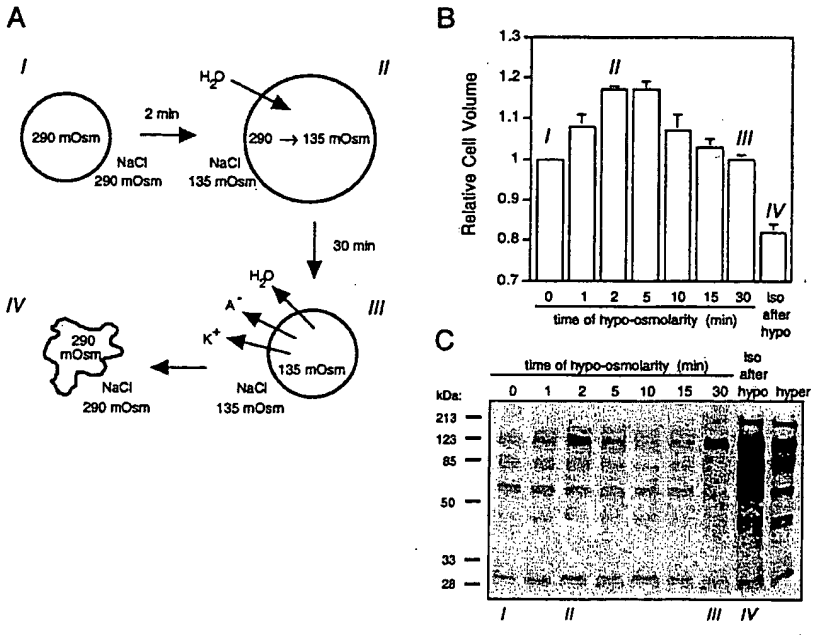


FIG. 6. **Effect of isotonic cell shrinkage on tyrosine phosphorylation.** *A*, schematic representation of the experimental protocols used to shrink cells under isotonic conditions. *I*, cells were initially in isotonic NaCl buffer; *II*, the cells were next suspended in isotonic sucrose medium at 4 °C and treated with 50 μg/ml nystatin for 10 min. This resulted in cell shrinkage; *III*, finally the shrunken cells were washed free of nystatin with fresh iso-osmotic sucrose buffer and then resuspended in warm (37 °C) isotonic NaCl medium. *B*, PMN were treated under the conditions indicated at the foot of the figure, using a protocol like that in *A*. The median volume of the cells was then measured using the Coulter Counter. The *roman numerals* in *B* and *C* identify the conditions with the diagram in *A*. Data are means ± S.E. of three experiments, normalized to the volume of untreated PMN in isotonic solution; *C*, cells were treated as in *B*, boiled in LSB, and tyrosine phosphorylation assessed by immunoblotting. *Lane 1*, isotonic control; *lane 2*, cells swollen by addition of nystatin to isotonic NaCl; *lane 3*, cells permeabilized with nystatin in medium with NaCl plus 50 mM sucrose, added to prevent excessive swelling; *lane 4*, cells shrunken by addition of nystatin to isotonic sucrose medium; *lane 5*, cells shrunken by addition of hypertonic NaCl medium (475 mOsm). Representative of three separate experiments.

some of the proteins that become tyrosine-phosphorylated when PMN shrink. The stimulation of NHE1 by growth factors has recently been reported to be partially dependent on the *erk1* and *erk2* MAPK ($p42/44^{MAPK}$) pathway (13). Moreover, it is well established that kinases of the MAPK family require phosphorylation on tyrosine residues to become active (24). Since hypertonic stress has been shown to induce the activation of *erk1* and *erk2* in other cell types (11, 13), we investigated whether these MAPK are the 40–42-kDa tyrosine-phosphorylated proteins observed in shrunken PMN. Cells were subjected to hypertonic stress for up to 30 min, and whole cell lysates were immunoblotted with an antibody that specifically recognizes the phosphorylated form of *erk1* and *erk2*. Fig. 9*A* shows that neither *erk1* nor *erk2* were tyrosine-phosphorylated in PMN in response to hypertonic stress. The sensitivity of the phospho-specific antibody and the responsiveness of the cells were assessed by stimulation with 100 nM fMLP, a well documented activator of *erk1* and *erk2* in PMN (25, 26). As shown in Fig. 9, comparable amounts of cell lysate revealed sizable amounts of phosphorylated *erk1* and *erk2* after treatment with the chemoattractant. We conclude that *erk1* and *erk2* are not phosphorylated during hypertonic challenge and are therefore unlikely to mediate the activation of NHE1.

Another member of the MAPK family, p38, has been shown to be activated by hypertonic stress in other cells (10) and was recently detected in fMLP-stimulated human PMN (17, 27). To test whether this kinase is phosphorylated and activated by shrinkage also in PMN we immunoprecipitated p38 and blotted the precipitates with anti-phosphotyrosine antibodies (Fig. 10*A*). Unlike other cells, PMN did not show evidence of p38 phosphorylation upon shrinking. As before, the effectiveness and sensitivity of the procedure were confirmed in parallel samples stimulated with fMLP (*rightmost lane* in Fig. 10*A*). That p38 was activated by chemoattractant but not by osmotic challenge was also confirmed in experiments where whole cell lysates were blotted with an anti-MAPKAPK-2 antibody (Fig. 10*C*). This kinase, a substrate of p38, undergoes an upward shift in electrophoretic mobility when phosphorylated (17). A distinct shift was noted for fMLP-stimulated samples but not in osmotically shrunken cells. We conclude that p38 is not phos-

FIG. 7. **Isotonically induced cell shrinkage following regulatory volume decrease: effect on tyrosine phosphorylation.** *A*, schematic representation of the protocol used to produce cell shrinkage in an isotonic buffer. *I*, PMN were initially suspended in isotonic NaCl buffer; *II*, cells were induced to swell in hypotonic (135 mOsm) NaCl medium; *III*, after 30 min, the cells had re-attained near-normal volume, through regulatory volume decrease; *IV*, iso-osmolarity was restored by addition of 90 mM NaCl, causing the cells to shrink. *B*, the cell volume was measured in a Coulter Counter at various time points following the resuspension of PMN in a hypotonic NaCl buffer. After 30 min, at which point the cell volume was similar to the initial volume, iso-osmolarity was restored by addition of 90 mM NaCl and after 5 min the cell volume was reassessed (iso after hypo). The *roman numerals* in *B* and *C* identify the conditions with the diagram in *A*. Data are means ± S.E. of three experiments, normalized to the volume of untreated PMN in isotonic solution; *C*, cells were treated as in *B*, boiled in LSB, and tyrosine phosphorylation assessed by immunoblotting. For comparison, cells were shrunken in hypertonic NaCl medium (475 mOsm; *rightmost lane*). Results are representative of three separate experiments.



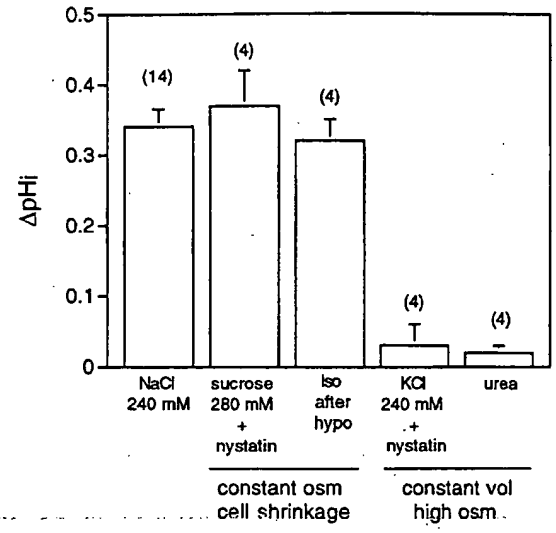


FIG. 8. **Dissociation of the effects of increased osmolarity and cell shrinkage on pH_i.** PMN were loaded with BCECF, and pH_i was determined fluorimetrically as described under "Experimental Procedures." The difference between the maximal pH_i, attained 5 min after application of the indicated treatment, and the basal pH_i is illustrated. *First bar*, cells suspended in isotonic medium were transferred to hypertonic NaCl. *Second bar*, PMN were shrunken by permeabilization with nystatin in iso-osmotic sucrose (10 min at 4 °C), washed free of nystatin, and transferred to warm (37 °C) isotonic NaCl buffer. *Third bar*, PMN were suspended in hypotonic NaCl buffer and allowed to regulate their volume for 30 min. Next, they were induced to shrink by transfer to isotonic NaCl medium. *Fourth bar*, PMN were nystatin-permeabilized in isotonic KCl buffer at 4 °C. Extra KCl (125 mM) was then added to render the medium hypertonic. Nystatin was then washed away, and finally, the cells were suspended in hypertonic NaCl medium; *fifth bar*, PMN were suspended in a NaCl medium made hyperosmotic by addition of 200 mM urea. Values of ΔpH_i are the means ± S.E. of the number of experiments indicated in *parentheses*.

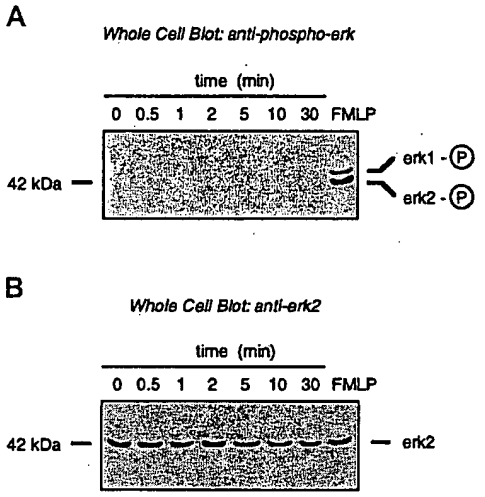


FIG. 9. **Effect of hypertonicity on the phosphorylation of *erk1* and *erk2*.** PMN suspended in isotonic NaCl buffer (time 0) were subjected to hypertonic stress by addition of an extra 100 mM NaCl to the medium. Samples were taken at the indicated times, subjected to SDS-PAGE, and transferred to PVDF membranes, which were used for immunoblotting. For comparison, a sample of cells treated with 100 nM fMLP (*FMLP*) for 1 min at 37 °C is also included. *A*, samples probed with an antibody that specifically recognizes the phosphorylated form of *erk1* and *erk2*. *B*, the membrane used in *A* was stripped and re-probed with an anti-*erk2* antibody, showing equal protein loading of all lanes. Results shown are representative of three separate experiments.

phorylated or activated by hypertonic challenge in PMN.

Hypertonic stress activates SAPK in a number of cells (*e.g.* Ref. 9). To investigate if SAPK was similarly stimulated in PMN, this kinase was precipitated from cell lysates using GST-c-*jun*-coupled to Sepharose beads and its activity tested *in vitro*. SAPK failed to phosphorylate GST-c-*jun* following hypertonic stress in PMN (results not shown). It is unclear whether SAPK is not activated or not expressed by human PMN, since we were also unable to demonstrate activation upon treatment of these cells with anisomycin, a well known activator of SAPK.

Src Family Kinases—Kinases of the *src* family are themselves regulated by phosphorylation on tyrosine residues and may account for the phosphotyrosine accumulation in the 60-kDa range in shrunken PMN. We therefore investigated the ability of cell shrinkage to induce the phosphorylation and activation of three *src* family kinases that are comparatively abundant in PMN, namely *fgr* (59 kDa), *hck* (56/59 kDa), and *lyn* (59 kDa). PMN were osmotically stimulated for 1 min and lysed, and the three tyrosine kinases were individually immunoprecipitated. The immunoprecipitates were subsequently separated by SDS-PAGE and blotted with a phosphotyrosine-specific antibody. As shown in Fig. 11*A*, all three kinases were significantly phosphorylated in untreated cells, and cell shrinkage promoted increased tyrosine phosphorylation of *fgr* and *hck*, whereas a slight decrease was noted for *lyn*. The effect of volume changes on the activity of these kinase assays was also tested, performing *in vitro* assays with immunoprecipitates from control and shrunken cells. We assessed the ability of the kinases to autophosphorylate as well as to phosphorylate the exogenous substrate enolase. Consistent with the phosphotyrosine immunoblots of Fig. 11*A*, both auto-phosphorylation and enolase kinase activity increased for *fgr* and *hck* but decreased slightly for *lyn* (Fig. 11*B*).

The identity of other tyrosine-phosphorylated proteins was also probed using sequential immunoprecipitation and blotting as in Fig. 11*A*. We failed to detect tyrosine phosphorylation of paxillin (67 kDa) or c-*cbl* (120 kDa) in PMN stimulated hypertonically (results not shown).

DISCUSSION

PMN are exposed to a wide range of dynamic physical forces during their active life span, particularly during passage through narrow capillaries and across vascular walls and during chemotaxis. Such mechanical stress causes shape and volume alterations that need to be compensated in order for the cells to function optimally (28). Such regulation of shape and volume can occur in part via the movement of ions and osmotically obliged water across the cell membrane. The current study investigated the mechanism that regulates the activation of a major, volume-sensitive ion transporter in human PMN, namely NHE1. The salient observations were (i) that a moderate reduction of the cell volume (≈16%) induced the tyrosine phosphorylation of several proteins and (ii) that such tyrosine phosphorylation is seemingly required for the activation of NHE1.

Several hypotheses exist regarding the mechanism(s) whereby cells detect osmotic stress (reviewed in Refs. 29–31). First, cells may sense the ionic strength or total osmolarity of the medium or of the intracellular milieu. This explanation cannot account for the observed phosphotyrosine accumulation in PMN for several reasons. Tyrosine phosphorylation could be induced by shrinkage at constant osmolarity and ionic strength (Figs. 6 and 7). Moreover, increasing the osmolarity and ionic strength at constant volume had minimal effect on phosphotyrosine formation (Figs. 4 and 5). It has also been suggested that changes in cytoskeletal architecture upon shrinking may mediate activation of the cells. While we cannot dismiss this

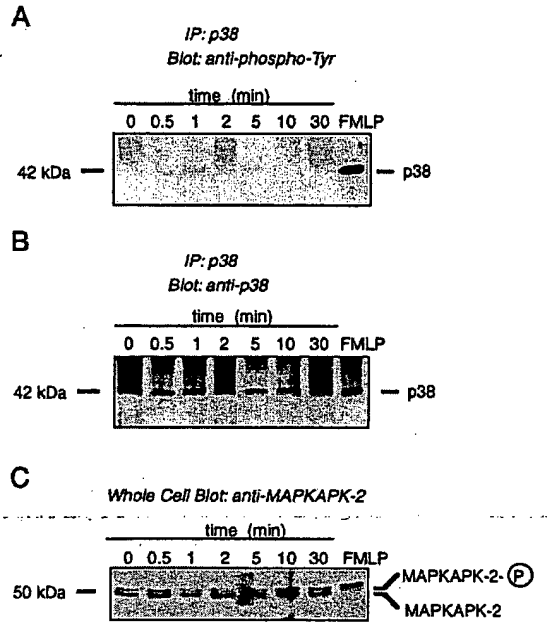


FIG. 10. **Effect of hypertonicity on the tyrosine phosphorylation and activity of p38.** *A*, PMN suspended in isotonic NaCl buffer (time 0) were subjected to hypertonic stress by addition of an extra 100 mM NaCl to the medium. Samples were taken at the indicated times and subjected to immunoprecipitation (*IP*) with anti-p38 antibodies, and the precipitates were used for immunoblotting with anti-phosphotyrosine (*anti-phospho-Tyr*) antibodies. For comparison, a sample of cells treated with 100 nM fMLP (*FMLP*) for 1 min at 37 °C is also included. *B*, the membrane used in *A* was stripped and re-probed with an anti-p38 antibody, showing comparable efficiency in all immunoprecipitates. *C*, PMN were subjected to a hypertonicity or FMLP as in *A*. Whole cells were then subjected to SDS-PAGE, and proteins were transferred to PVDF membranes, which were immunoblotted with anti-MAPKAPK-2 antibody. The mobility of the normal and phosphorylated (*P*) forms of MAPKAPK-2 are indicated. Results shown are representative of three separate experiments.

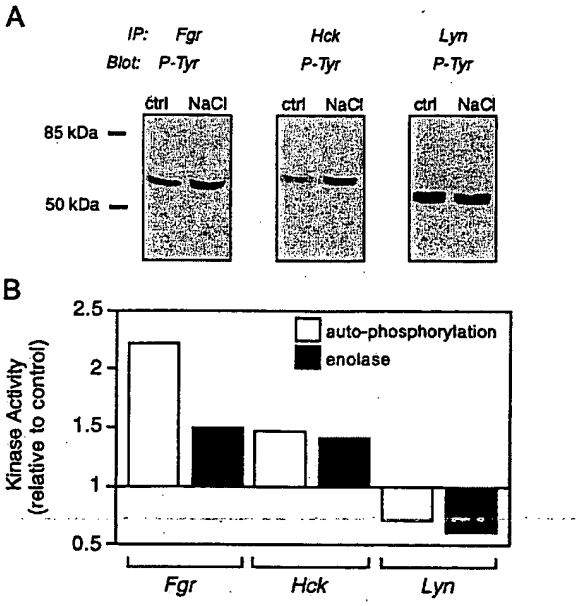


FIG. 11. **Effect of hypertonicity on the tyrosine phosphorylation and activity of *fgr*, *hck*, and *lyn*.** *A*, PMN suspended in isotonic NaCl were subjected to hypertonic stress (475 mOsm) for 1 min. After lysis in Nonidet P-40 buffer, *fgr*, *hck*, and *lyn* were immunoprecipitated (*IP*) with their respective antibodies as described under "Experimental Procedures." The immunoprecipitates were subjected to SDS-PAGE, transferred to PVDF membranes, and probed with a phosphotyrosine-specific (*P-Tyr*) antibody. Results shown are representative of three separate experiments. *B*, immunoprecipitates obtained as in *A* were probed for kinase activity in a medium containing 5 μg of enolase and 10 μCi of [^{32}P]ATP. Following kinase assays, the samples were subjected to SDS-PAGE, and the phosphorylation of enolase and the autophosphorylation of the kinases were quantified using a PhosphorImager. Results shown are the means of two experiments.

possibility, our data suggest that assembly of microtubules and *de novo* F-actin polymerization are not essential, since neither colchicine nor cytochalasin B prevented the volume-induced tyrosine phosphorylation (results not shown).

An interesting hypothesis stipulates that cells perceive their volume by sensing macromolecular crowding (29); small changes in cell volume can lead to large increases in the thermodynamic activity of macromolecules (32). One form of crowding, leading to such disproportionate increases in activity, may be the aggregation of surface receptors recently reported by Rosette and Karin (33). These authors found that osmotic shrinkage of HeLa cells induced clustering of interleukin-1, epidermal growth factor, and tumor necrosis factor receptors despite the absence of their ligands. Clustering of receptors is known to be crucial to their activation (34), and accordingly, receptor stimulation was found in the shrunken HeLa cells (33). In PMN, engagement and cross-linking of Fc receptors or of integrins lead to the activation of the tyrosine kinases *fgr* and *hck* (35–38), which were also found to be stimulated osmotically in this study. It is tempting to speculate that shrinkage of PMN induces the activation of *fgr* and *hck* through clustering of Fc receptors, integrins, and/or other tyrosine kinase (associated) receptors.

The similarity in the pattern of osmotic activation of tyrosine phosphorylation and of NHE1, together with the inhibitory effects of genistein and erbstatin, suggests that stimulation of tyrosine kinases precedes and is necessary for activation of ion exchange. A causal relationship between these events has in fact been postulated for several cell types (*e.g.* Ref. 39) including PMN where phagocytic stimuli (14) and chemotactic peptides (40) regulate pH_i in a tyrosine kinase-dependent manner. In the context of macromolecular crowding, it is noteworthy that cross-linking of Fc receptors and integrins can in fact activate NHE1 in PMN and in other cells (14, 41, 42). It is, however, unlikely that NHE1 itself is the target of the tyrosine kinases for the following reasons. First, only serine residues have been found to be phosphorylated in this isoform (2, 3). Second, in Chinese hamster ovary cells no increase in the phosphorylation of NHE1 was detected following activation by osmotic stress (6). Therefore, other intervening steps are likely situated between the tyrosine kinases and NHE1. Potential regulators of NHE1 include Ca^{2+}/calmodulin, protein kinase C, phosphatidylinositol 3-kinase, and heterotrimeric G proteins (43–46). We found, however, that depletion of Ca^{2+} had no effect on either tyrosine phosphorylation or NHE1 activation in response to hypertonic stimulation. Moreover, pretreatment of PMN with *bis*-indolylmaleimide (a protein kinase C inhibitor), wortmannin (a phosphatidylinositol 3-kinase inhibitor), or pertussis toxin (a heterotrimeric G protein inhibitor) all failed to inhibit NHE1 or the tyrosine phosphorylation stimulated by hypertonic stress.[3]

Hooley *et al.* (44) demonstrated that RhoA was involved in the activation of NHE1 in fibroblasts. Interestingly, a connection between tyrosine kinases and RhoA had been previously established (47). It is therefore conceivable that the pathway

[3] E. Krump, unpublished observations.

leading to osmotic activation of NHE1 involves stimulation of RhoA through *src*-related tyrosine kinases. The mechanism by which RhoA activates NHE1 is currently unknown, but some information can be gleaned from the recent identification of Rho-binding proteins. Of relevance, the phosphorylation of myosin light chain was found to be regulated by a RhoA-dependent kinase (48). This observation is important in that Shrode *et al.* (49) demonstrated that inhibitors of myosin light chain kinase were potent blockers of the osmotic activation of NHE1. One can therefore envisage the following sequence: cell shrinkage may lead to receptor clustering and activation of tyrosine phosphorylation. This would in turn activate Rho leading to stimulation of NHE1, possibly via phosphorylation of the light chain of myosin. It is noteworthy that MAPKs are seemingly not components of this signaling cascade.

In conclusion, the current study showed that the shrinkage of PMN induced the tyrosine phosphorylation of several proteins, two of which were identified as *fgr* and *hck*. Given the ability of tyrosine kinase inhibitors to block the stimulation of NHE1, we propose that tyrosine kinases, including *fgr* and *hck*, are involved in the osmotic activation of the antiporter, through some as yet unidentified intermediate(s).

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